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Angiotensin II modulates cell growth-related events and synthesis of matrix proteins in renal interstitial fibroblasts

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Angiotensin II modulates cell growth-related events and synthesis of matrix proteins in renal interstitial fibroblasts. The renin-angiotensin system seems to play an important role in the pathogenesis of renal interstitial fibrosis. However, the potential direct effects of angiotensin II (Ang II) on cultured renal fibroblasts have been little studied. We have observed that rat renal interstitial fibroblasts (NRK 49F cell line) possess AT_1 receptors coupled to intracellular calcium mobilization. Exposure of these cells to Ang II induced several short and long growth-related metabolic events mediated by the AT_1 receptor, including c-*fos* gene expression, changes in cell cycle and cell proliferation. Activation of interstitial fibroblasts by Ang II could also contribute to extracellular matrix accumulation. Stimulation with Ang II increased mRNA expression of TGF-β1, fibronectin and type I collagen. In fact, Ang II enhanced fibronectin production via AT_1 receptors by a process depending on autocrine TGF-β secretion. The mechanism of some Ang II actions (calcium mobilization and fibronectin production) depended on protein kinase C and tyrosine kinase activation. We further investigated whether renal fibroblasts could express some components of the renin-angiotensin system. These cells constitutively expressed the angiotensinogen gene that was up-regulated by Ang II. Collectively, these results indicate that in renal interstitial fibroblasts Ang II causes hyperplasia and extracellular matrix production via the AT_1 receptor. Ang II may initiate a positive feedback regulation of fibroblasts growth, inducing the expression of TGF-β1 and angiotensinogen genes. Ang II, acting directly on interstitial fibroblasts, may be implicated in the pathogenesis of renal fibrosis.

Fibroblasts are the main effector cells of renal interstitial fibrosis. Under normal conditions only a small proportion of these cells participate in the synthesis and deposition of matrix proteins, mainly type I collagen [1]. However, in pathological conditions, fibroblasts proliferate, migrate and synthesize growth factors and matrix proteins [2]. The development of interstitial fibrosis has been attributed to the release of cytokines by infiltrating mononuclear cells and tubular interstitial cells. These cytokines, acting on fibroblasts, could induce proliferation, recruitment of mononuclear cells and extracellular matrix synthesis [3, 4].

Recent data suggest that the renin-angiotensin system could play a certain role in the pathogenesis of glomerular and interstitial fibrosis [5–7]. Treatment with angiotensin converting enzyme inhibitors and angiotensin receptor antagonists decreased tubulointerstitial lesions in several models of renal injury [8–12]. Activation and redistribution of some components of renin-angiotensin system have also been demonstrated in some of these models, suggesting that this system could be associated with cell growth changes and sclerosis [13–15]. Moreover, interstitial changes have been observed in hypertension-induced renal injury and in *in vivo* systemic Ang II infusion, both situations characterized by mononuclear cell recruitment, proliferation of interstitial cells, and deposition of matrix proteins leading to renal interstitial fibrosis [16, 17]. However, a direct effect of Ang II on renal interstitial fibroblasts independently from secondary hemodynamic actions, which could explain the above-described processes, has not been studied.

Angiotensin II may act as a growth factor for several renal cells. In mesangial cells and tubular epithelial cells this peptide promotes growth changes and synthesis of extracellular matrix proteins. Some of these effects seem to be mediated by the release of growth factors, such as transforming growth factor-β (TGF-β) [7, 18]. TGF-β plays an important role in the pathogenesis of renal diseases [19] and has widespread effects on the turnover of extracellular matrix proteins [20].

In this work we have studied the potential effect of Ang II on growth-related cellular events in renal interstitial fibroblasts. To test this hypothesis, we first characterized whether renal fibroblasts express Ang II receptors and the intracellular signaling evoked by their stimulation. Secondly, we evaluated whether Ang II regulates fibroblast proliferation and the synthesis of extracellular matrix and TGF-β, as well as the mechanisms and the receptor type involved in these phenomena. Finally, we tested whether these cells could contribute to local Ang II generation focusing on angiotensinogen mRNA expression.

METHODS

Materials

All culture reagents were purchased from Gibco BRL (Paisley, Scotland, UK). Ang II was obtained from Calbiochem. Dup753 (AT_1 receptor antagonist) was provided by Dupont (Madrid, Spain) and PD123177 (AT_2 receptor antagonist) by Parke Davis (Barcelona, Spain). [^{35}S]-Methionine (1000 Ci/mmol), α-[^{32}P]dCTP (3000 Ci/mmol), α-[^{32}P]ATP (1200 Ci/mmol) and ^{125}I-[Sar^1,Ile^8]Ang II were purchased from Amersham (Buckinghamshire, UK). Primers for RT-PCR studies were obtained from Genosys Biotechnology (Cambridge, England, UK). Polyclonal

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anti-fibronectin (FN) antibody was obtained in rabbits by immunization against human FN [21]. Monoclonal mouse anti-TGF-β1 antibody and recombinant human TGF-β1 were obtained from Immugenex (Los Angeles, CA, USA). The rest of chemicals employed were from Sigma (St Louis, MO, USA).

Cell cultures

The cell line NRK 49F (ATCC:CRL1570; American Type Culture Collection, Rockville, MD; USA) is derived from rat kidney fibroblasts. Cells were grown in RPMI with 5% FCS (Gibco) at 37°C in the presence of 5% CO_2.

Determination of angiotensin II receptors

Receptor binding studies were performed on subconfluent monolayers of fibroblasts, cultured in 12-well plates, using ^{125}I-[Sar^1,Ile^8]Ang II. Cells were maintain for 18 hours in serum-free RPMI, and after, in RPMI with 0.5% (wt/vol) of BSA for one hour. Then cells were incubated with ^{125}I[Sar^1,Ile^8]Ang II (0.1 nM). Competition studies were performed using either Ang II or the receptor subtype-specific nonpeptide antagonist [22] DUP753 (AT_1 specific) or PD123177 (AT_2 specific) at various concentrations (10^{-12} to 10^{-5} M). Nonspecific binding was determined in the presence of 1000-fold excess of unlabeled Ang II and was less than 10% of the total cell bound radioactivity. Binding parameters (K_d and number sites/cells) were calculated using the Instat program. After incubation for three hours at 4°C (equilibrium binding achieved), cells were washed twice with ice-cold phosphate saline (PBS) to remove unbound compound. Then cells were solubilized in 1 M NaOH/0.1% Triton X-100. Radioactivity in cell lysates was determined in a gamma counter. Specific binding was calculated as the difference between total and nonspecific ligand bound.

Measurement of intracellular free calcium

Measurement of calcium mobilization was made as previously described [23]. Monolayer cells grown on 13 mm diameter crystal coverslips were incubated in serum-free medium during the last 18 hours before the experiment. The cells were then washed and loaded with the fluorescence Ca^{2+} indicator fura-2 acetometoxy ester (2 μM) in RPMI-HEPES for 60 minutes at 37°C, and incubated again for 30 minutes in a fura-2-free medium for intracellular dye cleavage. Cells were then rinsed twice with Krebs-Hensleit-HEPES buffer (KHH) (130 mM NaCl, 5.3 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 20 mM HEPES, 5.5 mM glucose, and 0.5% BSA, pH 7.3), and coverslips were mounted in a quartz cuvette with 1 ml of KHH buffer at 37°C. Substances prewarmed in KHH buffer were added directly to the cuvette. Changes in intracellular calcium ($[Ca^{2+}]_i$) were continuously monitored by recording changes in the fluorescence ratio at 510 nm, with the excitation at 340/380 nm in a spectrofluorometer at 37°C (model LS-50; Perkin-Elmer Corp., Norwalk, CT, USA). In some experiments, extracellular Ca^{2+} was depleted from the medium by addition of 3 to 5 mM EGTA just before the fluorescence analysis. In these experiments, after addition of Ang II (10^{-7} M) and when baseline returned to control levels, Ca^{+2} was replaced in the medium to 2 mM of final concentration. To characterize intracellular signal systems, cells were preincubated with the protein kinase C (PKC) inhibitor, staurosporine (10^{-6} M, 30 min), or the tyrosine kinase inhibitor, genistein (10^{-5} M, 60 min). To determine the angiotensin receptor type involved in calcium response, cells were preincubated with DUP753 (AT_1 specific; 10^{-6} M, 30 min) or PD123177 (AT_2 specific; 10^{-5} M, 30 min).

Cell-cycle analysis by fluorescence-activated cell sorting

For determination of cell size and DNA content, subconfluent cells grown in 75 cm^2 petri dishes were incubated in a serum-free medium for 48 hours to make them quiescent. Then cells were incubated during 24 hours with Ang II (10^{-7} M) and 10% FCS as the positive control. At the end of the incubation period, the cells were washed three times with PBS and trypsin. After centrifugation, the cellular pellet was treated with 100 μg/ml ribonuclease A and DNA was stained with 100 μg/ml propidium iodide in 0.005% Nonidet P-40. The stained specimen was kept in the dark at 4°C before flow cytometry. Cells were sieved to remove dead cells and/or cellular debris. Data were analyzed with the EPICS-C flow cytometer (Coulter, Hialeah, FL, USA) using the PARA-1 program [24] and represented as either contour plots or histograms. The percentage of cells in the Go, S and G_2 + M phases, mean channel number and coefficient of variance (CV) for relative size and DNA content were obtained from histograms after computer analysis.

Measurement of cellular proliferation

The effect of Ang II on cell proliferation was determined by methylene blue assay [25]. Quiescent subconfluent cells, grown on 96-well plates, were incubated for 24, 48 and 72 hours in serum-free medium with daily doses (every 24 hr) of Ang II (range 10^{-7} to 10^{-11} M). For experiments with Ang II receptor antagonists, cells were preincubated with DUP753 (10^{-6} M, 30 min) or PD123177 (10^{-5} M, 30 min) before the addition of 10^{-7} M Ang II. After the incubation period, cells were washed with PBS and fixed. Cells were stained with methylene blue in 0.01 M Borate buffer. The absorbance was measured at 650 nm in a microplate photometer.

RNA isolation, Northern blot and PCR assays

The expression of transcripts for AT_1 receptor, c-*fos*, extracellular matrix proteins and TGF-β1 was determined using Northern blot analysis. The angiotensinogen gene expression was studied by a semiquantitative RT-PCR technique.

Northern blot analysis. Quiescent confluent fibroblasts, grown in 75 cm^2 petri dishes, were stimulated in a serum-free medium. After the incubation period, total RNA was extracted by the Chomczynski and Sacchi method [26] and quantitated by absorbance at 260 nm. Northern blot analysis was performed as previously described [27]. The cDNA probes used were: c-*fos* (ATCC#), (α1)I (Hf677) and (α1)IV (pCVIV-1-PE16) collagens, 28S, rat FN (SR270) and murine TGF-β1, obtained as previously described [27], and labeled with α-[^{32}P]dCTP. The riboprobe of the AT_1 receptor (kindly provided by Dr. M. Martincz-Maldonado, originally cloned by Murphy et al [28]) was used as the antisense cRNA from linearized plasmid with *Hin*dIII using SP6 promotor and labeled with α-[^{32}P]-ATP. Blots were prehybridized for four hours at 42°C in hybridization solution (50% formamide, 1% SDS, 5 × SSC, 1× Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA and 50 mM sodium phosphate buffer, pH 6.5) and hybridization was carried out at 42°C overnight with 20% dextran sulfate and [α-^{32}P]-denatured probe. The filters were washed using a 2 × SSC, 0.1% SDS, at room temperature for 30

minutes and then twice with 0.2 × SSC, 0.1% SDS, at 55°C for 15 minutes. For the AT_1 receptor, prehybridization and hybridization were carried out at 60°C in modified solution (hybridization mixture with 8× Denhardt's solution and 1 mM EDTA), and the filters were washed first at 65°C and finally at 70°C, in the same conditions as described above. Autoradiography was performed using standard methods.

Reverse transcription and semiquantitative PCR analysis. Total RNA was reverse transcribed to single-stranded cDNA by incubation with a reverse transcription mixture (5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTPs mixture, 20 U rRNAsin ribonuclease inhibitor, 15 U AMV reverse transcriptase and 50 ng random primers) at 42°C for 30 minutes following the instructions of the manufacturer (Promega). PCR analysis for angiotensinogen and Glyceraldehyde 3'-phosphate dehydrogenase (G3PDH) was conducted in the following conditions (1 min at 63°/54°C, respectively, to allow annealing of the primers, 3 min at 72°C for primer extension, and 1 min at 94°C to denature the double-stranded DNA). The following primers were used for rat angiotensinogen [29]: (antisense) 5'-CCAGCCGG-GAGGTGCAGT-3' and (sense) 5'-TTCAGGCCAAGACCT-CCC-3', that yielded products of 308 base-pair. G3PDH was used as an internal control and the following primers were used [30]: (antisense) 5'-ATACTGTTACTTATACCGATG-3' and (sense) 5'-AATGCATCCTGCACCACCAA-3' that yielded products of 515 bp. Both amplifications were done for 20, 30, 35, and 40 cycles in order to establish the linearity of the reaction. In all experiments, the presence of possible contaminants was checked by control reactions in which amplification was carried out in complete reaction mixture lacking template DNA or with RNA samples from RT reactions done in the absence of AMV reverse transcriptase. The DNA products from the PCR reactions were analyzed on a 1.5% agarose gel or a 4% polyacrylamide/urea gel in Tris-borate EDTA buffer. The gels were dried and exposed for autoradiography.

Autoradiograms were scanned using the Image Quant densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Results were expressed as arbitrary densitometric units relative to 28S intensity bands (Northern blot) and G3PDH (RT-PCR).

Determination of fibronectin synthesis

New fibronectin (FN) synthesis was measured by metabolic labeling with [^{35}S]-Methionine and immunoprecipitation with anti-FN antibodies [31]. Quiescent cells grown in 24 well plates, were incubated in methionine-free culture medium RPMI with 20 μCi/ml of [^{35}S]-methionine and stimuli. After 24 hours, medium was collected and cells were lysed with extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 2 mM EDTA and 5 mM NEM, pH 7.4). Aliquots of the supernatants (diluted 1:10 in extraction buffer) and cell lysates were immunoprecipitated with an excess of anti-FN antibody (50 μg) for 16 to 18 hours at 4°C. After this incubation, immune complexes were recovered by addition of protein A-Sepharose beads (Pharmacia, Uppsala, Sweden), and incubated for one additional hour. Beads were washed in extraction buffer. FN was released by heating at 100°C for five minutes in electrophoresis sample buffer and analyzed under denaturing conditions in 7.5% SDS-polyacrylamide gels. The gels were handled by fluorography techniques and autoradiography was performed by standard methods. In order to normalize FN production to cell number, samples aliquots (20 μl) of cell lysates were taken for determination of DNA content. As a negative control for immunoprecipitation, isotopic normal rabbit IgG (30 μg/ml) was used.

For experiments with Ang II receptor antagonists, cells were preincubated with DUP753 (10^{-6} M, 30 min) or PD123177 (10^{-5} M, 30 min). To further investigate the mechanism of FN synthesis, cells were treated with the inhibitors of RNA and protein synthesis, actinomycin D (Act D; 50 μg/ml) and cycloheximide (CHX; 1 μg/ml), respectively, in control and Ang II-stimulated cells (10^{-7} M Ang II, added 30 min after the inhibitors). To characterize intracellular signal systems, cells were preincubated for 30 minutes with the inhibitor of PKC, staurosporine (Staur.; 10^{-8} M) or tyrosine kinase, genistein (Genist.; 10^{-6} M) before the addition of 10^{-7} M Ang II. To determine the role of endogenous TGF-β, cells were coincubated with 10 μg/ml of anti-TGFβ neutralizing antibody and 10^{-7} M Ang II. Recombinant human TGF-β (50 pM) was employed as positive control for FN synthesis.

Statistical analysis

Results are expressed as the mean ± SEM. Significance was established using a Student's *t*-test and analysis of variance. Differences were considered significant if the *P* value was less than 0.05.

RESULTS

Renal interstitial fibroblasts possess AT_1 receptors

We first examined whether rat renal interstitial fibroblasts (NRK cell line) express Ang II receptors. Binding studies were performed by using 0.1 nM ^{125}I-[Sar^1,Ile^8]Ang II in subconfluent cells. In preliminary studies we determined binding time and temperature. Binding of ^{125}I-[Sar^1,Ile^8]Ang II increased linearly for up to one hour and remained constant for an additional six hours at 4°C. At this temperature there was no internalization of the complexes, a process that was found at 37°C (data not shown). The competition binding of ^{125}I-[Sar^1,Ile^8]Ang II by unlabeled Ang II indicated the existence of a specific binding in renal interstitial fibroblasts with a K_d of 0.23 nM and around 54000 sites/cell (Fig. 1A). Ang II receptors have been described in medullary interstitial cells [32] and in rabbit renal interstitial fibroblasts [33]. In interstitial mice fibroblasts (TFB cell line, [34]), we also determined the Ang II binding sites showing similar results to those described for rat cells (data not shown). Moreover, the affinity of these receptors (K_d of 0.23 nM) in rat renal fibroblasts was similar to that reported for AT_1 receptors in fibroblasts of different tissues (cardiac [35, 36] and skin [37]), in vascular smooth muscle cells [38] and mesangial cells [39].

To determine the receptor subtype present in renal fibroblasts, competition studies were made in the presence of two Ang II specific nonpeptidic receptor antagonists (DUP753, AT_1 receptor antagonist and PD123177, AT_2 receptor antagonist). Fibroblasts were incubated with ^{125}I-[Sar^1,Ile^8]Ang II and increasing concentrations of DUP753 and PD123177 for three hours at 7°C (Fig. 1A). The AT_1 receptor antagonist, DUP753, completely displaced the bound labeled compound. By contrast, the AT_2 receptor antagonist, PD123177, did not displace the labeled peptide, though at concentrations higher than 10^{-5} M a slight effect was noted. A similar effect has been observed in cardiac fibroblasts, but it was not clear whether this displacement of the radioligand

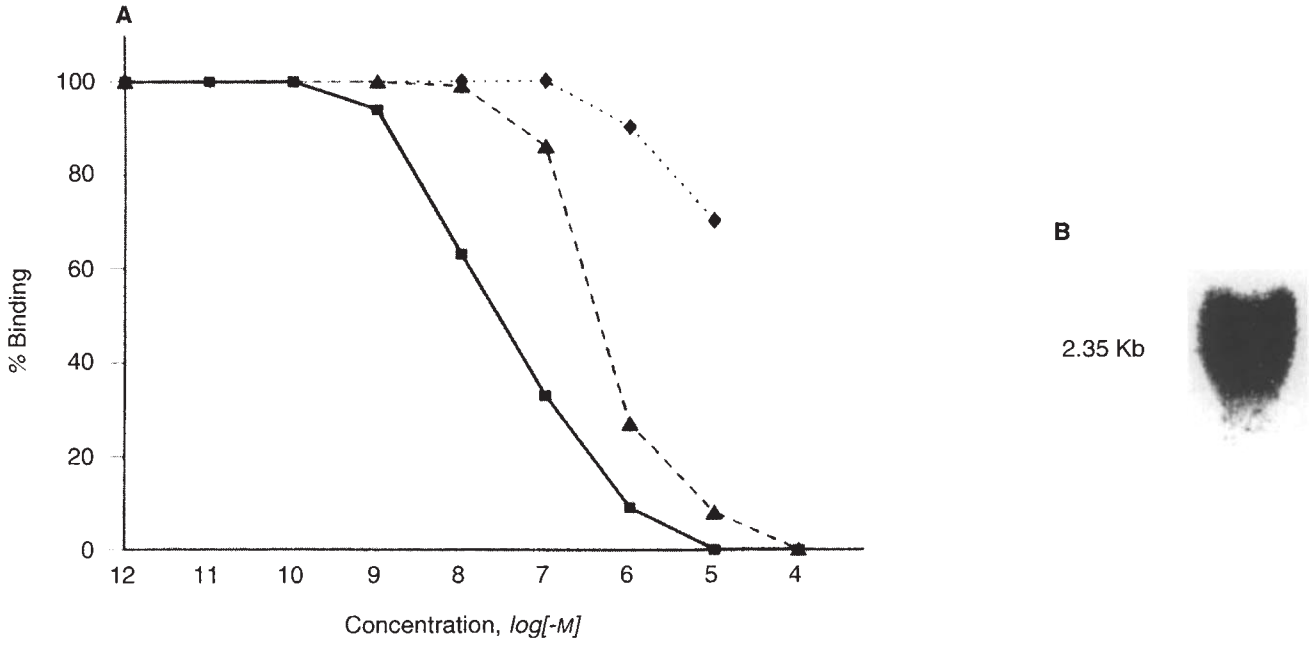

Fig. 1. Renal interstitial fibroblasts possess angiotensin II binding sites. (*A*) Displacement of ^{125}I[Sar^1,Ile^8]Ang II binding from renal fibroblasts. Competition of the binding of ^{125}I[Sar^1,Ile^8]AngII by increasing concentrations of unlabeled AngII (■), Dup753 (▲) or PD123177 (♦). Data represent mean ± SEM from 3 independent experiments made by duplicate. (*B*) Renal interstitial fibroblasts express AT_1 mRNA. Total RNA was extracted from unstimulated cells and hybridized with the AT_1 receptor riboprobe. Representative Northern blot showing that fibroblasts express a mRNA transcript of 2.35 Kb corresponding to the AT_1 receptor.

with high concentrations of PD123177 was a specific competition or a crossover interaction with the AT_1 receptor [35].

To confirm the presence of AT_1 receptors in rat renal interstitial fibroblasts, we determined whether these cells express AT_1 receptor mRNA. Total RNA was extracted and analyzed by Northern blot using a riboprobe for AT_1 receptor gene cloned from vascular smooth muscle cells [28]. Fibroblasts expressed a transcript of 2.35 kb (Fig. 1B), corresponding to AT_1 receptor mRNA described in vascular smooth muscle cells [28]. Together, these data indicate the presence of Ang II receptors in renal interstitial fibroblasts.

Angiotensin II receptors are linked to calcium mobilization

Angiotensin II increases calcium concentration in mesangial cells [40], tubular epithelial cells [41] and cardiac fibroblasts [36], though the intensity of the response varied among the cell types. Intracellular Ca^{+2} mobilization was determined by continuous monitoring of fluorescence intensity [23]. Cells were loaded with fura-2/AM and the emitted fluorescence in response to Ang II stimulation was measured. Angiotensin II (10^{-6} to 10^{-11} M) evoked a rapid rise in intracellular free calcium. The transient increase in Ca^{2+} was dramatic at higher concentrations of Ang II (Fig. 2).

The next point of our study was to investigate the mechanism of calcium mobilization in response to Ang II. After chelation of extracellular Ca^{2+} by EGTA, Ang II (10^{-7} M) still elicited an increase in intracellular Ca^{+2} concentrations (Fig. 3), though the decay to resting levels was faster than in the presence of extracellular Ca^{2+}. When the baseline returned to control levels, Ca^{2+} was replaced in the medium (2 mM of final concentration) and an increase in fluorescence was observed, probably due to the influx

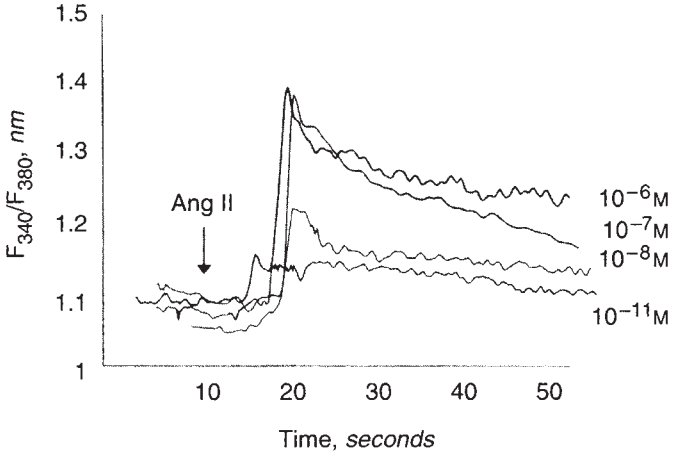

Fig. 2. Stimulation of interstitial fibroblasts with angiotensin II induces calcium increase in a dose-dependent manner. Cells in cover slips were loaded with fura-2 and stimulated with Ang II (10^{-6} to 10^{-11} M). Continuous fluorescence monitoring of $[Ca^{2+}]_i$ levels was performed. Values are ratios of emitted fluorescence at 340/380 nm excitation. A representative experiment of 6 to 9 experiments is shown.

of Ca^{2+} from the extracellular environment. These data suggest that the transient increase in intracellular Ca^{2+} may result from both mobilization of intracellular Ca^{2+} stores and Ca^{2+} influx across the membrane. The observation that Ang II stimulated a calcium transient event in the absence of extracellular calcium suggests that Ang II could mobilize calcium from intracellular stores via inositol thriphosphate and therefore activate phospholipase C, presenting the signaling characteristic of Ang II responses in several cell types [7, 35, 36, 40].

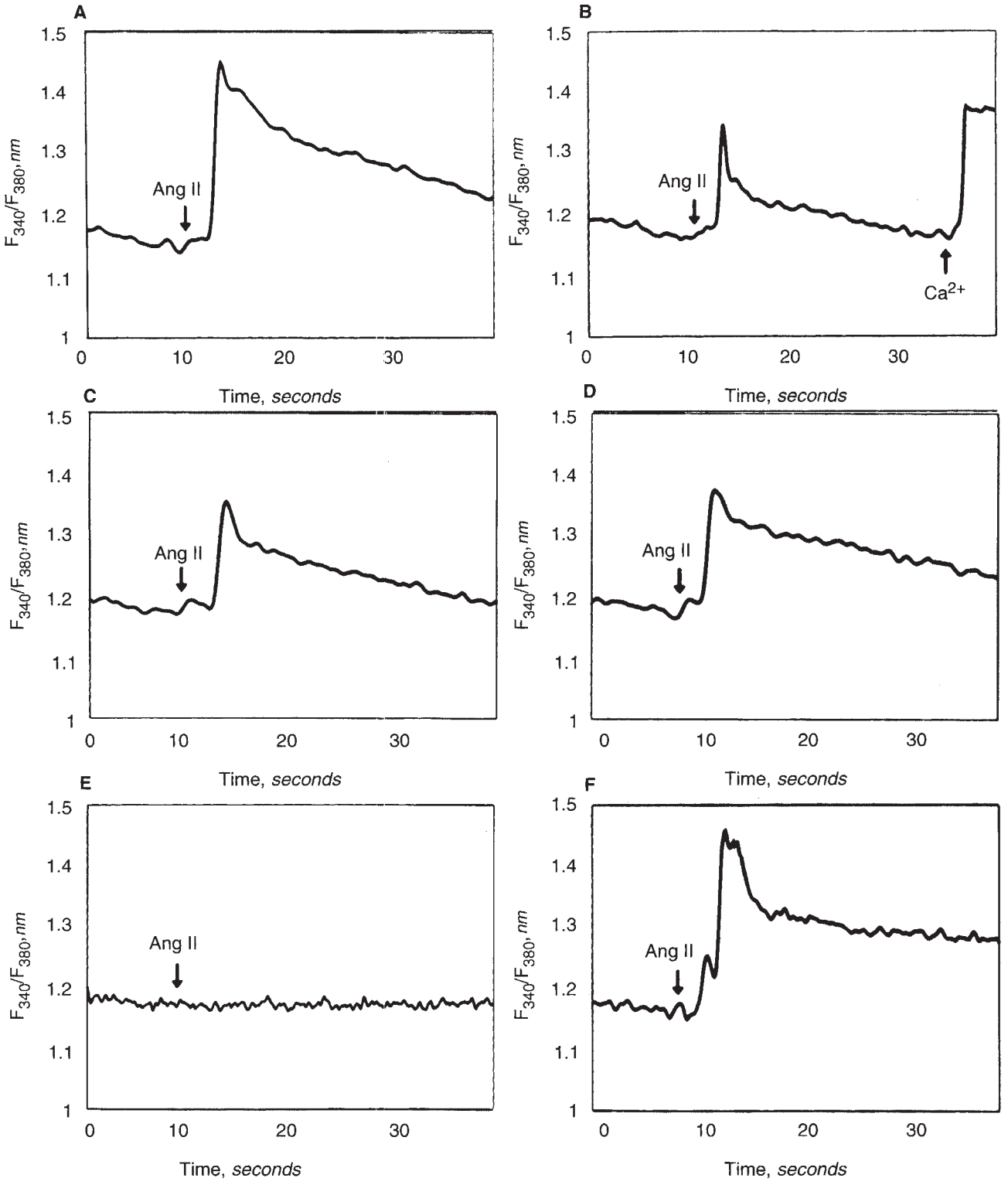

Fig. 3. Molecular characterization of calcium mobilization in angiotensin II-stimulated fibroblasts. Control and pretreated cells were loaded with fura-2 and fluorescence measurements after addition of 10^{-7} M Ang II were performed. Experimental details are given in the **Methods** section. (*A*) In control cells 10^{-7} M Ang II increased $[Ca^{2+}]_i$. (*B*) When extracellular Ca^{2+} was depleted from the medium by addition of EGTA, a reduction in Ang II-induced Ca^{2+} increase was observed. When $[Ca^{2+}]_i$ returned to control levels, a final concentration of 2 mM Ca^{2+} was added (indicated by an arrow). Effect of PKC (C) and tyrosine kinase (D) inhibitors. Role of AT_1 receptors; in the presence of DUP753 (AT_1 antagonist), Ang II-induced Ca^{2+} mobilization was blocked (E), while no effect was observed with PD123177 (AT_2 antagonist) (F).

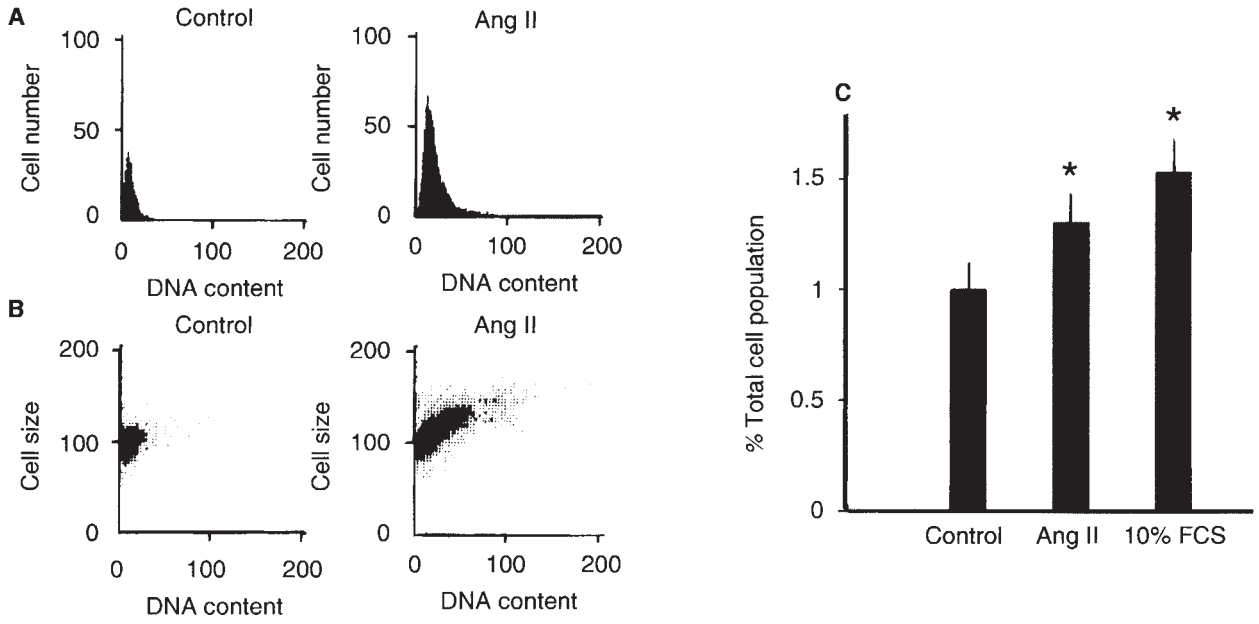

Fig. 4. Cell cycle analysis of angiotensin II-stimulated renal interstitial fibroblasts. Treatment of rested cells for 24 hours with 10^{-7} M Ang II. DNA was stained with propidium iodide after RNase treatment. Resulting scans are expressed as contour plots. Histogram (*A*) shows the relationship of the DNA content (x-axis) versus cell number (y-axis) and (*B*) shows the DNA content (x-axis) versus cell size (y-axis). (*C*) The percent of cells in $S+G_2/M$ in relation to total cell population. As a positive control 10% FCS was used. Results are expressed as mean ± SEM of 3 experiments. $^*P < 0.05$ versus control.

Other second messenger pathways, elicited after Ang II transmembrane signaling, include activation of PKC and tyrosine kinase phosphorylation [42, 43]. For this reason, we wondered whether the activation of these kinases was involved in the calcium mobilization yielded by Ang II in renal interstitial fibroblasts. Cells were preincubated for 30 minutes with the inhibitors of PKC and tyrosine phosphorylation, staurosporine (10^{-6} M) and genistein (10^{-5} M), respectively, before the measurement of Ca^{+2} mobilization. Treatment with both agents did not affect cell viability. As shown in Figure 3, both inhibitors caused a marked reduction in Ca^{2+} rise in Ang II-stimulated cells (65% and 55% vs. Ang II alone, respectively, $N = 6$, $P < 0.05$). Therefore, these results suggest that activation of PKC and tyrosine phosphorylation are involved in Ang II-induced calcium mobilization.

To determine the receptor type involved in Ang II-induced Ca^{2+} response, cells were preincubated for 30 minutes with AT receptor antagonists, DUP753 (10^{-6} M) and PD123177 (10^{-5} M). The presence of AT_1 antagonist blocked Ang II-induced Ca^{+2} mobilization (Fig. 3), while the AT_2 antagonist did not affect the kinetics of Ca^{2+} mobilization. These results suggest that AT_1 receptors presented in renal interstitial fibroblasts are coupled to intracellular calcium mobilization.

Angiotensin II elicits cell hyperplasia, but not hypertrophy, via AT_1 receptors

Angiotensin II is now considered to be a renal growth factor, however, its effects on cell growth depend on the cell type studied. Ang II induced hypertrophy in tubular epithelial cells [18], while most authors found a hyperplasic response in mesangial cells [7]. Thus, we were interested in whether Ang II could modulate the growth in renal interstitial fibroblasts.

By flow cytometry analysis (FACS), we determined the variation of the cell cycle and cell size induced by Ang II. To obtain a quiescent state (G_o), cells were maintained for 48 hours in serum-free medium. After treatment with 10^{-7} M Ang II for 24 hours we observed changes in cellular DNA content that reflected progression through the S phase and mitosis (Fig. 4A). Ang II-treated cells presented a high percentage of cells in mitosis ($G_2/M + S > G_o/G_1$; Fig. 4 A, C). However, the cell size did not vary in response to the Ang II stimulation (control, 20 ± 10; Ang II, 24 ± 11; mean channel ± coefficient of variance, $N = 3$; P = NS; Fig. 4B). These data show that when Ang II was added to quiescent (G_o) renal fibroblasts, cells entered G_1 and progressed to S phase.

To further evaluate the effects of Ang II on fibroblast growth, the methylene blue assay was performed. Treatment of growth-arrested fibroblasts, in a serum-free medium, with 10^{-7} M Ang II for 48 hours and 72 hours significantly increased cell proliferation (Fig. 5A). This effect was dose-dependent between 10^{-7} M to 10^{-9} M Ang II (Fig. 5B) with an optimal concentration of 10^{-7} M (167 ± 12% over control, $N = 4$, $P < 0.05$, that represents 30% with respect to that observed with 10% FCS, which was used as the positive control). These results suggest that Ang II causes hyperplasia, but not hypertrophy, in renal interstitial fibroblasts, in the conditions of our study.

Next, we examined the Ang II receptor subtype linked to the proliferative response. Preincubation for 30 minutes with the AT_1 receptor antagonist DUP753 (10^{-6} M), and subsequent stimulation with 10^{-7} M Ang II for 48 hours, caused a 70% inhibition in cell proliferation compared to Ang II alone ($N = 4$, $P < 0.05$). By contrast, the AT_2 receptor antagonist PD123177 (10^{-5} M) did not exert any effect (Fig. 5C). Neither DUP753 nor PD123177 alone significantly affected cell proliferation in nonstimulated cells (Fig.

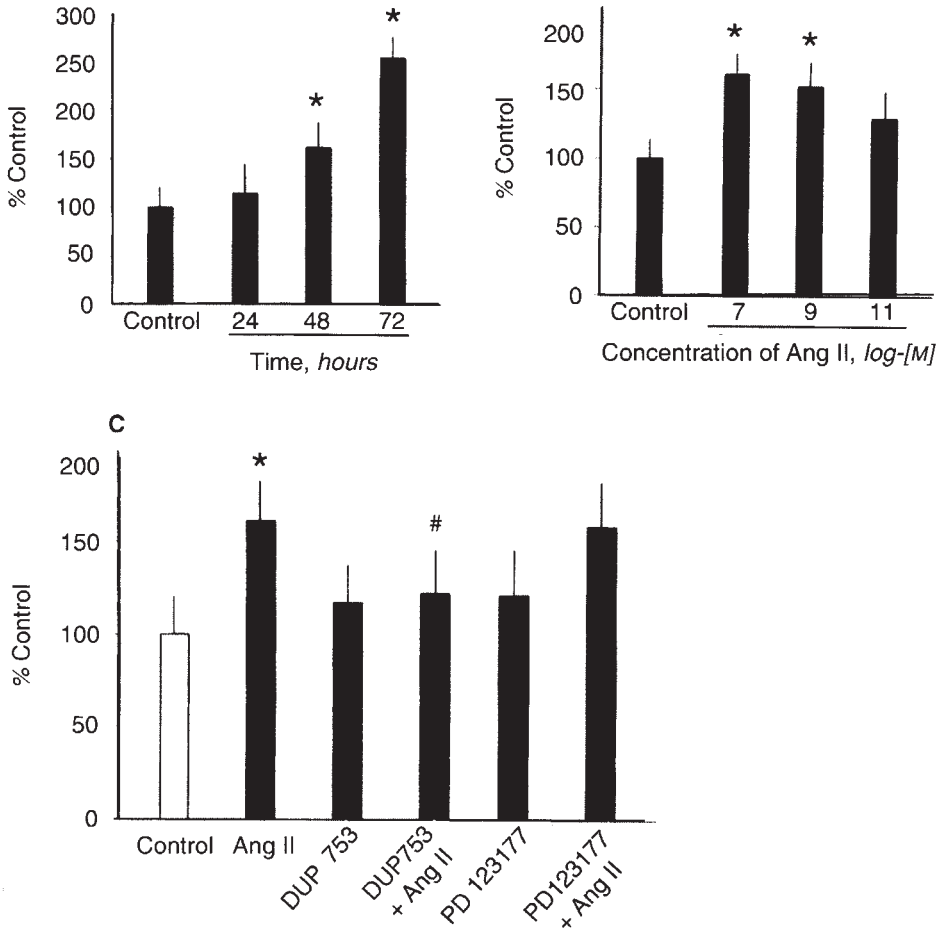

Fig. 5. Mitogenic effect of angiotensin II in renal interstitial fibroblasts. (*A*) Time course of 10^{-7} M Ang II-stimulation. (*B*) Dose response after 48 hours of incubation. (*C*) Effect of AT_1 (DUP753) and AT_2 (PD123177) receptor antagonist on Ang II-stimulated (10^{-7} M) cells after 48 hours. Cell proliferation was determined by methylene blue assay. Bars represent mean ± SEM of 4 experiments made in triplicate. $^*P < 0.05$ versus control; $\#P < 0.05$ versus Ang II alone.

5C). These results suggest that Ang II-induced cell proliferation is mediated by the AT_1 receptor.

Angiotensin II induces the expression of growth-related nuclear proto-oncogene c-*fos*

Rapid induction of nuclear proto-oncogenes, such as c-*fos*, is one of the earliest transcriptional events, and has been associated with cell proliferation, cellular differentiation, and hypertrophy [44]. The c-*fos* protein forms part of the AP-1 complex that binds to the transcription control elements of some genes [45]. *In vivo* systemic Ang II-infusion induces c-*fos* gene expression in the rat kidney [46]. In our study, quiescent renal interstitial fibroblasts were stimulated with 10^{-7} M Ang II for one hour. Afterwards, RNA was isolated and c-*fos* mRNA expression analyzed by Northern blot. Ang II induced a c-*fos* transcript of 2.2 Kb, while this gene did not appear in cells in quiescent state (G_0) (Fig. 6). Preincubation with the AT_1 receptor antagonist (10^{-6} M), but not the AT_2, abolished Ang II-induced gene expression (Fig. 6), suggesting that an AT_1 receptor-dependent mechanism is involved.

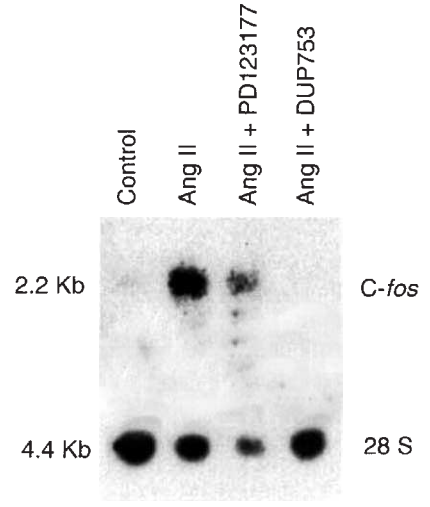

Fig. 6. Angiotensin II induces c-*fos* mRNA expression in renal interstitial fibroblasts through AT_1 receptors. Cells were stimulated for one hour with 10^{-7} M Ang II in control conditions or pretreated for 30 minutes with AT_1 (DUP753) or AT_2 (PD123177) receptor antagonists. Then, RNA was extracted and c-*fos* gene expression was determined by Northern blot. The figure shows a representative experiment of 2 that were done.

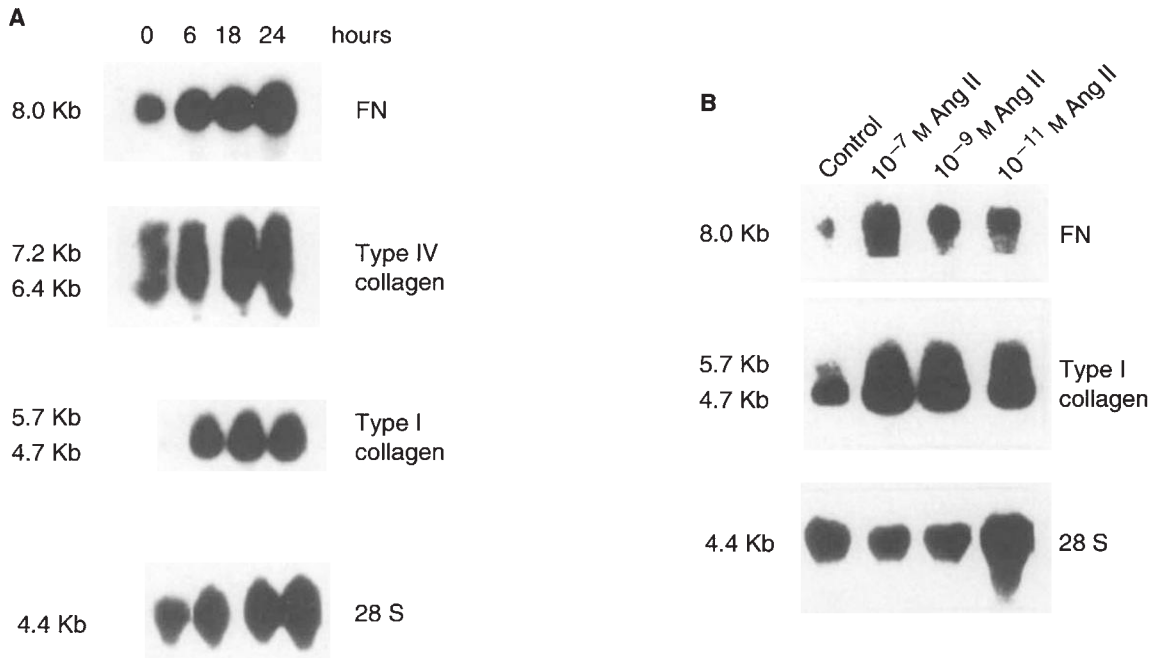

Fig. 7. Angiotensin II increases extracellular matrix gene expression in renal interstitial fibroblasts. (*A*) Time-course evolution of Ang II action. Cells were incubated for 0 to 24 hours with 10^{-7} M Ang II. (*B*) Dose-response of Ang II stimulation after 24 hours. The membranes were hybridized with cDNA probes of fibronectin (FN), type IV collagen [(α1)IV], type I collagen [(α1)I] and 28S. A representative Northern blot of 3 blots is shown.

Angiotensin II stimulates gene expression of extracellular matrix proteins

Many reports have demonstrated that fibroblasts are a major source of extracellular matrix [3]. We initially evaluated the effect of Ang II on the message levels of some matrix proteins. Stimulation of growth-arrested fibroblasts with Ang II for 18 to 24 hours increased the steady-state mRNA levels of fibronectin (FN) and type I collagen compared to control cells. These effect was dose-dependent, with a maximal response at 10^{-7} M Ang II (2.5- and 1.5-fold increase vs. control, after 18 and 24 hr, respectively). By contrast, there was no effect on type IV collagen mRNA expression (Fig. 7).

Angiotensin II increases fibronectin production, via protein kinase C and tyrosine kinases

We assessed the changes in FN production by metabolic labeling with [^{35}S]-Methionine and immunoprecipitation with anti-FN antibodies. We have observed that most of the FN produced by renal fibroblasts in normal conditions is secreted to the extracellular medium (around 70% of total FN). Treatment of resting fibroblasts with Ang II (10^{-7} to 10^{-11} M) for 24 hours caused a significant increase in FN production, with a maximal response at 10^{-7} M Ang II (165 ± 12% increase vs. control 100% ± 11, $N = 9$; $P < 0.05$; Fig. 8A). In addition, the relationship between soluble and cell-associated fraction was modified showing an increase in cellular FN production (control, 70% soluble vs. 30% cell-associated; Ang II, 47% vs. 53%).

In adult rat cardiac fibroblasts, Ang II appears to stimulate collagen synthesis by both AT_1 and AT_2 receptors [47]. Therefore, we also evaluated the receptor involved in Ang II-induced FN production. As shown in Figure 8B, preincubation with the AT_1 receptor antagonist DUP753 (10^{-6} M), but not with the AT_2 receptor antagonist PD123177 (10^{-5} M), inhibited Ang II-induced FN synthesis (DUP753, 95% inhibition vs. Ang II alone, $N = 6$, $P < 0.05$; PD123177, 30% inhibition, $N = 6$, P = NS). Neither DUP753 nor PD123177 alone significantly affected FN synthesis (Fig. 8B). These results suggest that Ang II regulates matrix accumulation through the AT_1 receptor.

To elucidate the mechanism of Ang II effect on FN production, we studied the role of the *de novo* RNA and protein synthesis. As shown in Figure 8C, pretreatment of cells with actinomycin D (ActD, 50 μg/ml) and cycloheximide (CHX, 1 μg/ml), inhibitors of RNA and protein synthesis, respectively, markedly reduced the amount of FN in control conditions and in response to 10^{-7} M Ang II stimulation (approximately 65% and 70% inhibition vs. control, respectively, $N = 3$, $P < 0.05$). These data suggest that FN synthesis induced by Ang II in renal interstitial fibroblasts is regulated at the transcriptional level.

To clarify the intracellular mechanism of Ang II effects on FN synthesis, we determined the role of some signaling events. We wondered whether activation of PKC and tyrosine were involved. Recently, it has been demonstrated that PKC activation can induce FN production [48]. Also, Ang II activates PKC in several cell types [40, 42]. In renal interstitial fibroblasts, treatment with the phorbol ester PMA, an activator of PKC, increased FN production (176 ± 15% increase vs. control, $N = 3$, $P < 0.05$; Fig. 8D). The preincubation with the inhibitor of PKC, staurosporine (Staur., 10^{-8} M) for one hour, significantly diminished ($N = 3$, $P < 0.05$) the Ang II-induced FN production (Fig. 8D). Treatment with staurosporine (10^{-8} M) did not affect cell viability. These results indicate that Ang II increases FN through a PKC dependent pathway.

We also studied the role of tyrosine kinases in fibroblasts in response to Ang II. Cells were preincubated for 30 minutes with the inhibitor of tyrosine kinase, genistein (Genist, 10^{-6} M), before

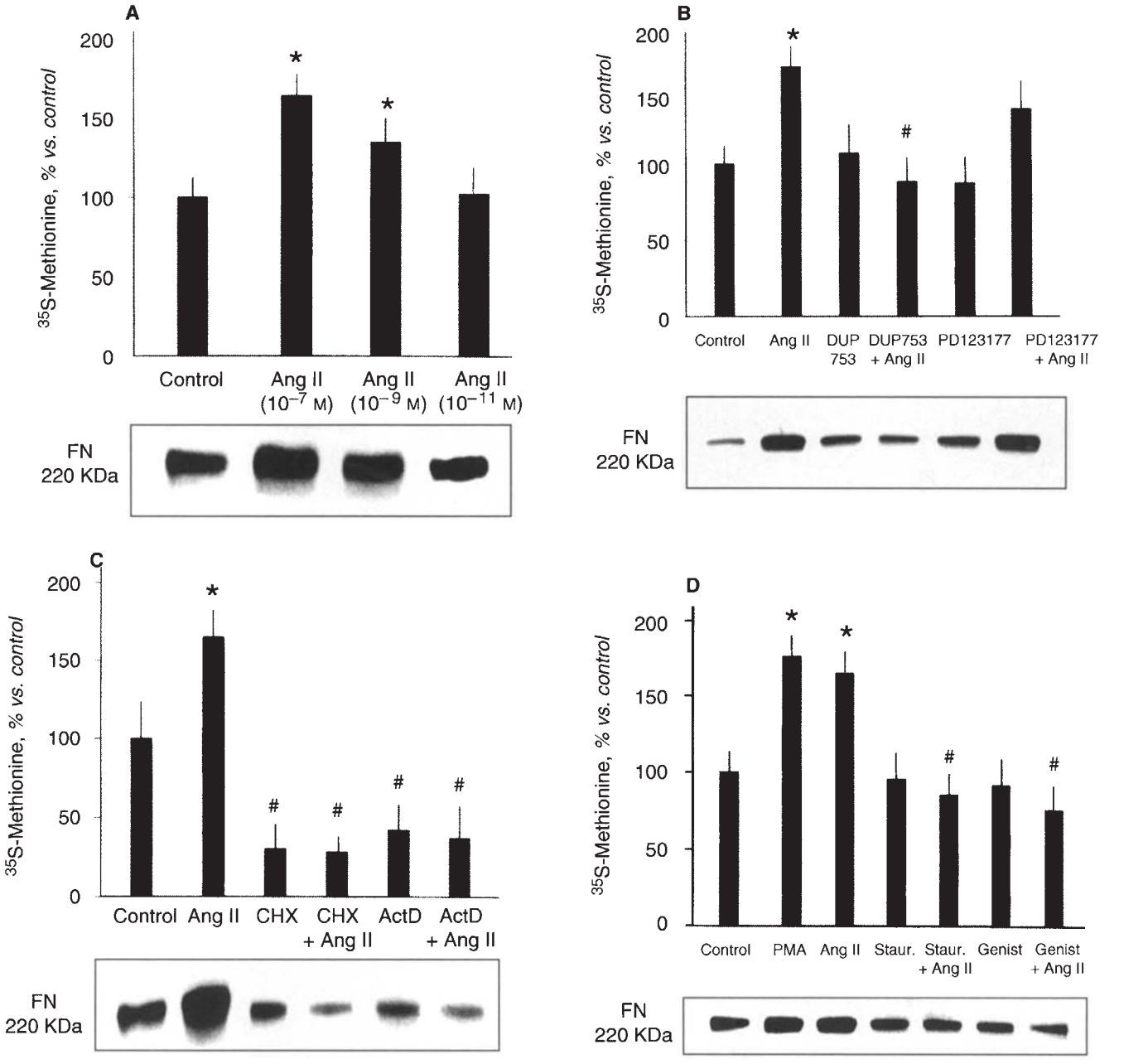

Fig. 8. Fibronectin synthesis induced by angiotensin II in renal interstitial fibroblasts. Cells were metabolically labeled with ^{35}S-Methionine and stimulated for 24 hours. Experimental details are given in the **Methods** section. (*A*) Dose-response of Ang II stimulation. (*B*) Effect of AT_1 (DUP753) and AT_2 (PD123177) receptor antagonist on Ang II-stimulated cells. (*C*) Effect of the inhibitors of protein and RNA synthesis (CHX is cycloheximide; ActD is actinomycin D) on Ang II. (*D*) Role of PKC and tyrosine kinase inhibitors (staur. is staurosporine, and genist., genistein) in Ang II-induced FN production. Upper panels show the densitometric analysis of the FN-bands and the lower panels show a representative autoradiography of PAGE-SDS of FN. Data are shown as percentage of increase versus control in arbitrary units, and are the mean ± SEM of 6 to 9 experiments. *$P <$ 0.05 versus control. #$P < 0.05$ versus Ang II.

Ang II stimulation. As shown in Figure 8D, tyrosine kinase inhibition caused a significant reduction in FN synthesis ($N = 3$, $P < 0.05$). These result suggest that tyrosine phosphorylation may participate in Ang II-induced FN production.

Role of TGF-β in matrix synthesis elicited by angtiotensin II

Since TGF-β is the major cytokine involved in matrix regulation [20], we tested the hypothesis that endogenous TGF-β may be responsible for the FN production observed upon Ang II stimulation.

We first examined the effect of Ang II on TGF-β expression by Northern blot analysis. Stimulation of quiescent cells with 10^{-7} M Ang II increased the mRNA levels of TGF-β1 with a maximal peak at six hours of incubation (2.3-fold; Fig. 9A). The response to Ang II was markedly attenuated by the AT_1 receptor antagonist DUP753 (Fig. 9B). Stimulation of renal interstitial fibroblasts with

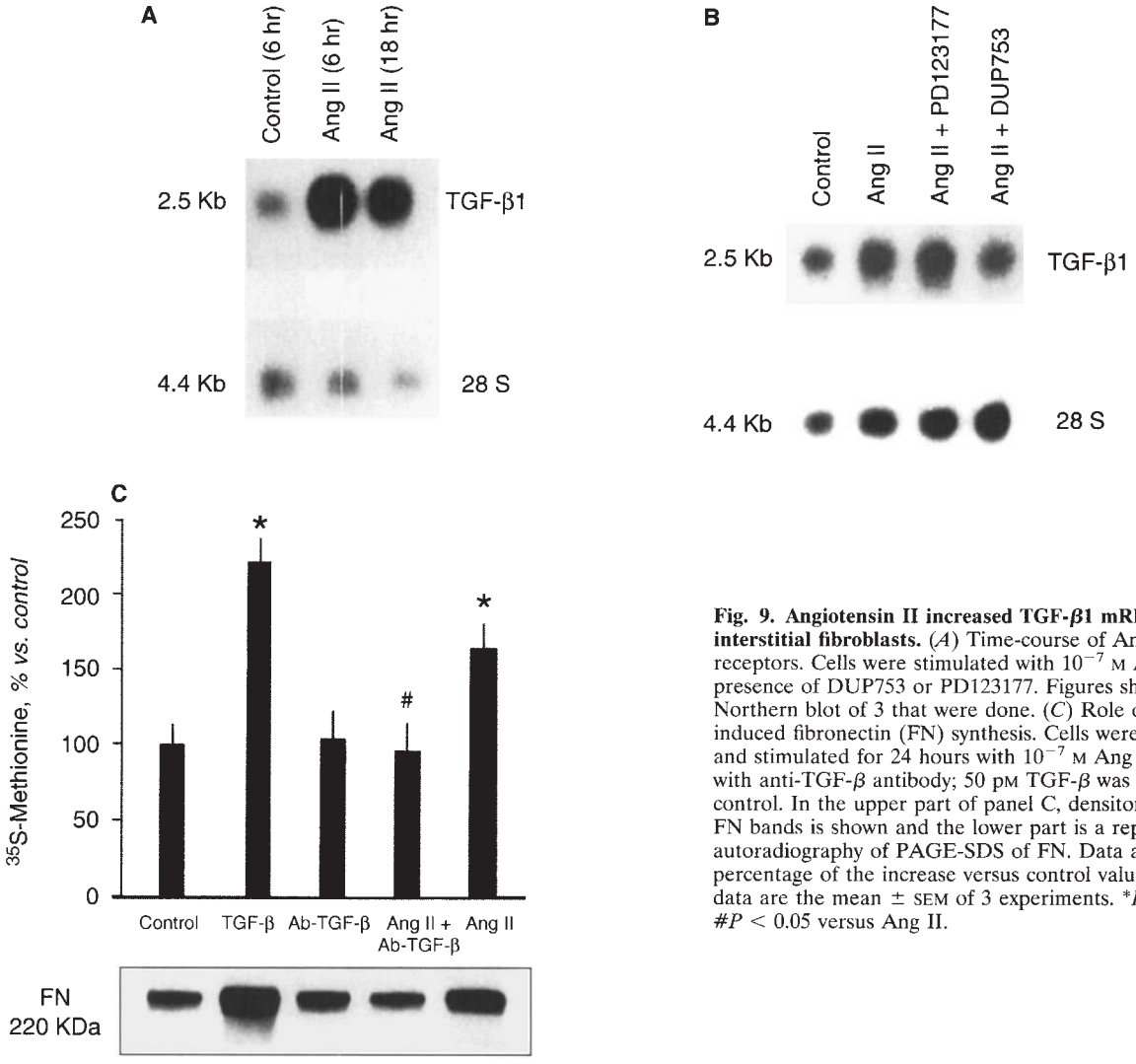

Fig. 9. Angiotensin II increased TGF-β1 mRNA expression in renal interstitial fibroblasts. (*A*) Time-course of Ang II. (*B*) Role of AT_1 receptors. Cells were stimulated with 10^{-7} M Ang II alone or in the presence of DUP753 or PD123177. Figures show a representative Northern blot of 3 that were done. (*C*) Role of TGF-β in Ang II-induced fibronectin (FN) synthesis. Cells were metabolically labeled and stimulated for 24 hours with 10^{-7} M Ang II alone or coincubated with anti-TGF-β antibody; 50 pM TGF-β was used as the positive control. In the upper part of panel C, densitometric analysis of the FN bands is shown and the lower part is a representative autoradiography of PAGE-SDS of FN. Data are shown as a percentage of the increase versus control values in arbitrary units; data are the mean ± SEM of 3 experiments. $^*P < 0.05$ versus control. $\#P < 0.05$ versus Ang II.

TGF-β (50 pM) increased FN production (220 ± 18% increase vs. control, $N = 3$, $P < 0.05$), with a higher response than Ang II (Fig. 9C). In the presence of neutralizing anti-TGFβ antibody (10 μg/ml), the Ang II-induced increase in FN production was totally abolished ($N = 3$, $P < 0.05$; Fig. 9C). This effect appears to be specific since normal rabbit IgG failed to inhibit the Ang II-stimulated FN synthesis (not shown). Neither anti-TGFβ antibody alone nor control IgG had any effect on FN synthesis. This result suggests that endogenous TGF-β mediates the Ang II effect on FN synthesis in renal interstitial fibroblasts.

Renal interstitial fibroblasts express angiotensinogen mRNA levels: Regulation by angiotensin II

By RT-PCR, we observed that interstitial fibroblasts expressed angiotensinogen mRNA in a constitutive manner (Fig. 10). This suggests that those cells could participate in the Ang II production in the kidney. It has been shown that Ang II increases angiotensinogen mRNA in rat liver [49] and in cardiac myocytes [50]. We have observed that angiotensinogen gene expression is up-regulated by Ang II (1.6-fold, after 6 hr; Fig. 10). This activation was completely inhibited by DUP753, but unaffected by PD123177 (Fig. 10).

DISCUSSION

In this paper we approached the idea that Ang II may participate in the pathogenesis of remodeling and fibrosis of renal interstitium by stimulating fibroblasts in a direct manner. The major findings of our work are: (*a*) Renal interstitial fibroblasts possess angiotensin receptors, mainly of the AT_1 subclass, linked to calcium mobilization. (*b*) Ang II induces growth-related events in fibroblasts such as hyperplasia, matrix accumulation and TGF-β synthesis through the AT_1 receptor. (*c*) The activation of PKC and tyrosine phosphorylation may be involved in Ang II signaling. (*d*) Renal interstitial fibroblasts express angiotensinogen mRNA, which is up-regulated by Ang II.

Angiotensin II binds to specific high-affinity receptors on the surface of responsive cells. Most of physiological responses of Ang II, like vessel contraction, calcium release and growth-related events, are mediated by the AT_1 receptor [51]. However, recent studies suggest that the AT_2 receptor is involved in the control of

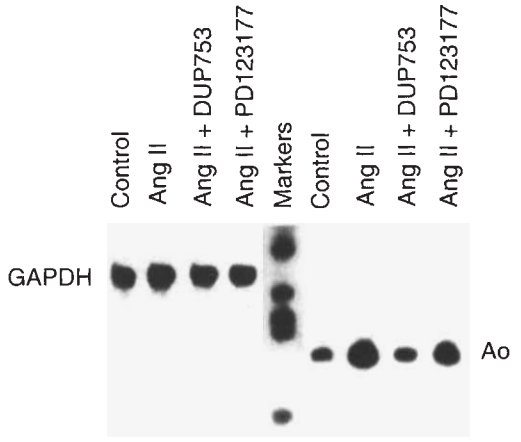

Fig. 10. Demonstration of angiotensinogen gene expression in renal interstitial fibroblasts. Renal fibroblasts constitutively express angiotensinogen (Ao) mRNA as shown by RT-PCR. Cells were stimulated for six hours with 10^{-7} M Ang II alone or in the presence of DUP753 or PD123177. A representative experiment of 3 RT-PCR is shown; G3PDH was used as the internal control.

cell differentiation [52], apoptosis [53], thropic effects on vascular smooth muscle cells [54], mononuclear cell recruitment [55, 56] and natriuretic response [57]. Moreover, an overexpression of AT_2 receptor has been observed during tissue damage [58]. In the adult kidney, the AT_1 receptor is present in mesangial, tubular and interstitial cells [7, 32, 39], while the AT_2 receptor is more abundant in fetal kidney [59]. In this paper we have observed that rat renal interstitial fibroblasts (NRK49F cell line) possess binding sites for Ang II, mainly of the AT_1 receptor subtype, and express the mRNA for this receptor. This AT_1 receptor presents a similar K_d to that found other cells, including fibroblasts of different tissues (cardiac and skin) as well as mesangial and vascular smooth muscle cells [33–39].

In general, the AT_1 receptor is linked to the calcium-phospholipid-dependent signaling pathway. In renal interstitial fibroblasts, we observed that the Ang II receptor is coupled to calcium release, both from intracellular stores and across the membrane, keeping a similar behavior to cardiac fibroblasts and mesangial cells, but different from tubuloepithelial cells and fetal skin fibroblasts [35–37]. In these latter cells, the AT_1 receptor stimulates cAMP production, in contrast to other cells in which it is coupled to adenyl cyclase in an inhibitory manner [60].

Several investigators, including ourselves [61], have observed that Ang II induces hyperplasia/hypertrophy in mesangial cells, while it causes only hypertrophy in tubular epithelial cells [7, 18]. In this study we have demonstrated that Ang II caused modification in the cell cycle in growth-arrested fibroblasts (serum-free medium), inducing the entry into G_1 phase. Also, after 48 hours of incubation Ang II significantly increased fibroblast proliferation mediated by AT_1 receptor. Furthermore, stimulation for one hour with Ang II induced c-*fos* gene expression, one of the earliest transcriptional events associated with cell growth [44]. Indirect evidence suggested that Ang II could modify the response of interstitial renal fibroblasts. The systemic infusion of Ang II into normal rats markedly up-regulated α-smooth muscle actin expression in the interstitium, indicating that Ang II may promote the phenotypic modulation of fibroblasts or expand the specific population of major fibroblasts [16, 17]. Our results therefore suggest that in renal interstitial fibroblasts, Ang II, via AT_1 receptors, activates short- and long-term growth-related metabolic events.

Interstitial fibrosis occurs in progressive renal diseases. The progression to end-stage renal failure is determined more by the degree of tubulointerstitial injury than the extent of glomerular damage. Fibroblasts from human kidneys with interstitial fibrosis proliferate and synthesize more collagen types I, III and V, and FN than fibroblasts from healthy kidneys [62]. Several studies suggest that Ang II may be involved in the accumulation of extracellular matrix in the kidney [63]. In this sense, *in vivo* infusion of Ang II causes interstitial fibrosis, characterized by deposition of collagens and FN [16, 17]. Also, the administration of angiotensin converting enzyme (ACE) inhibitors and AT_1 receptor antagonist reduces collagen and FN expression and synthesis in animal models of progressive renal disease [9–12]. There is, however, no evidence of a direct effect of Ang II on interstitial fibroblasts. Two different renal cells participate in the development of interstitial fibrosis, tubulo-epithelial cells and fibroblasts, being the latter the main effector cell of this process [2]. In cultured proximal epithelial cells Ang II induces hypertrophy and stimulates the synthesis of type IV collagen, but not type I collagen [41]. Our *in vitro* data show that the interaction of Ang II with renal interstitial fibroblasts increased cell proliferation and the steady state levels of extracellular matrix proteins (mainly FN and type I collagen). These results suggest that local Ang II, acting on both cell types, could contribute to interstitial fibrosis, characterized by tubular atrophy, fibroblast proliferation and matrix accumulation.

TGF-β plays a key role in the pathogenesis of renal injury, leading to fibrosis and renal dysfunction in several kidney diseases [19]. TGF-β contributes to extracellular matrix accumulation by increasing matrix production, inhibiting its degradation and modulating cell-integrin receptors [20]. An interrelationship between Ang II and TGF-β has been described [7]. *In vivo* Ang II infusion increased glomerular TGF-β production [64], and *in vitro* several cell types, including mesangial and tubular cells, respond to Ang II stimulation by increasing TGF-β expression and synthesis [64, 41]. Moreover, in experimental models of renal injury, the treatment with ACE inhibitors and AT_1 receptor antagonist diminished renal TGF-β expression [11, 12]. In this work we have demonstrated that in renal interstitial fibroblasts Ang II, via AT_1 receptors, increased TGF-β1 mRNA expression. Also, the fact that neutralizing antibody against TGF-β abolished the FN synthesis induced by Ang II in these cells suggests that the autocrine production of TGF-β is involved in Ang II-induced matrix accumulation.

The effects of Ang II on growth, gene expression and FN production are mediated primarily by AT_1 receptor. DUP753 completely blocked Ang II effects, whereas AT_2 receptor antagonist did not interfere with these Ang II responses. These results support recent data that AT_1 receptor antagonist are as effective as ACE inhibitors in reducing interstitial damage in renal disease [12], and suggest that this beneficial effect could be due to the modulation of Ang II responses (cell proliferation and synthesis of matrix proteins and TGF-β) in interstitial fibroblasts.

We further investigated some mechanisms in response to Ang II stimulation in renal interstitial fibroblasts. We have observed

that in these cells, AT_1 receptors are linked to calcium mobilization by a mechanism dependent of PKC and tyrosine kinase activation. Binding of Ang II to AT_1 receptor increases protein tyrosine phosphorylation and activates several protein kinases, such as PKC, MAP kinase, calcium calmodulin-dependent kinase and S6 kinase [42, 43]. Numerous studies suggest an important role for PKC as an intracellular mediator of the effects of some hypertrophic growth stimuli [65], including FN production [48]. In this work, we have observed that Ang II-induced calcium mobilization and FN production was inhibited by staurosporine, a PKC inhibitor, suggesting that activation of PKC is involved in Ang II responses. Specific phosphotyrosine-containing proteins have been identified as substrates for Ang II-stimulated tyrosine kinases, including PLC-γ1 [66] and STAT family of transcription factors [67]. Recent studies in vascular smooth muscle cells suggest that many Ang II effects require tyrosine phosphorylation, such as vessel contraction, inositol triphosphate formation, protein synthesis and MAP kinase activity, as shown by the employment of tyrosine kinase inhibitors [43, 68, 69]. In this work, we have shown that Ang II-induced calcium mobilization and FN synthesis were blocked by genistein, a tyrosine kinase inhibitor. Although the AT_1 receptor is a typical G protein-coupled receptor that lacks tyrosine kinase activity, these results suggest that a receptor-associated tyrosine-kinase may be involved in Ang II signaling. All these results contribute to elucidate the role of tyrosine kinases in Ang II actions in renal cells.

Another major finding of our study was that renal interstitial fibroblasts showed constitutive angiotensinogen mRNA, suggesting that those cells could possess intracellular renin-angiotensin system and therefore contribute to local Ang II generation. These data are in agreement with studies on cardiac fibroblasts that present intracellular Ang I, Ang II and ACE immunoreactivity [70]. Although future studies are necessary to determine whether all components of this system, like renin and ACE activity, are present in renal fibroblasts, increasing evidence suggests that these cells could contribute to local Ang II generation. We have also observed that angiotensinogen gene expression was up-regulated in response to Ang II, thus showing a positive feedback regulation. A similar phenomenon has previously been observed in hepatocytes and cardiac myocytes [50, 71]. Activation of the transcription factor NF-κB could be involved in the regulation of angiotensinogen mRNA levels in hepatocytes [72]. Recent data have shown the activation of transcription factors during tissue damage [73]. Also, elevated angiotensinogen mRNA levels have been observed in those situations [74]. In this sense, in a model of immune complex nephritis, we have recently demonstrated an increase in NF-κB activity in renal cortex [75] coincidentally with an increase in cortical ACE activity and an up-regulation of renal angiotensinogen mRNA expression [15]. In addition, we have also demonstrated that Ang II activates NF-κB in cultured glomerular mesangial cells and vascular smooth muscle cells [75, 76]. Therefore, it is reasonable to speculate that Ang II-induced angiotensinogen gene in renal interstitial fibroblasts could be mediated by activation of NF-κB. All these data suggest that these cells are not only a target of Ang II, but might also contribute to a sustained synthesis of local Ang II, and therefore potentially to further tissue damage.

In summary, in this study we have noted that Ang II caused hyperplasia of renal interstitial fibroblasts and increased synthesis of extracellular matrix, via the AT_1 receptor type. Furthermore, Ang II may initiate a positive-feedback regulation of fibroblasts growth, inducing the expression of TGF-β1 and angiotensinogen genes. These results suggest that locally increased Ang II generation, by direct interaction with fibroblasts, could contribute to the pathogenesis of interstitial fibrosis.

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